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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12P 17/04, 7/60

A1

(11) International Publication Number: WO 97/43433

(43) International Publication Date: 20 November 1997 (20.11.97)

(21) International Application Number: PCT/US97/08668

(22) International Filing Date: 16 May 1997 (16.05.97)

(30) Priority Data:

60/017,879	17 May 1996 (17.05.96)	US
08/845,295	25 April 1997 (25.04.97)	US

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(81) Designated States: AU, BR, BY, CA, CN, CZ, HU, IL, JP, KR, MX, NO, NZ, PL, RU, SG, SK, TR, UA, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ENZYMATIC PROCESS FOR THE MANUFACTURE OF ASCORBIC ACID, 2-KETO-L-GULONIC ACID AND ESTERS OF 2-KETO-L-GULONIC ACID

(57) Abstract

The present invention is directed toward efficient, high-yield processes for making ascorbic acid, 2-keto-L-gulonic acid, and esters of 2-keto-L-gulonic acid. The processes comprise reacting the appropriate starting materials with a hydrolase enzyme catalyst such as a protease, an esterase, a lipase or an amidase.

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ENZYMATIC PROCESS FOR THE MANUFACTURE OF ASCORBIC ACID, 2-KETO-L-GULONIC ACID AND ESTERS OF 2-KETO-L-GULONIC ACID

FIELD OF THE INVENTION

This invention relates to processes for the manufacture of ascorbic acid, 2-keto-L-gulonic acid (KLG), and esters of KLG. More particularly, the present invention relates to the use of enzyme catalysts in the manufacture of ascorbic acid, KLG or esters of KLG.

BACKGROUND OF THE INVENTION

Ascorbic acid, also known as vitamin C, is a dietary factor which must be present in the human diet to prevent scurvy and which has been identified as an agent that increases resistance to infection. Ascorbic acid is used commercially, for example, as a nutrition supplement, color fixing agent, flavoring and preservative in meats and other foods, oxidant in bread doughs, abscission of citrus fruit in harvesting and reducing agent in analytical chemistry.

One current method for the manufacture of ascorbic acid utilizes a modification of the original Reichstein-Grossner synthesis (Reichstein et al., *Helv. Chim. Acta*, *17*:311 (1934); U.S. Pat. No. 2,301,811 to Reichstein; all references cited herein are specifically incorporated by reference). In this process a glucose source is converted to ascorbic acid. During conversion an intermediate of a diacetonide of KLG is produced.

Several two stage methods exist for the manufacture of ascorbic acid. In the first stage, glucose is converted via fermentation processes to either an isolated intermediate of KLG (Sonoyama et al., *Applied and Envtl. Microbiology*, *43*:1064-1069 (1982); Anderson et al., *Science*, *230*:144-149 (1985); Shinjoh et al., *Applied and Envtl. Microbiology*, *61*:413-420 (1995)) or the intermediate of the Reichstein-Grossner synthesis, the diacetonide of KLG.

The second stage, which converts either of the intermediates to ascorbic acid, proceeds by one of two reported routes. The first route, a modification of the latter steps of the Reichstein-Grossner synthesis, requires a multitude of steps whereby the intermediate is esterified with methanol under strongly acidic conditions to produce methyl-2-keto-L-gulonate (MeKLG). The MeKLG is then reacted with base to produce a metal ascorbate salt. Finally, the metal ascorbate salt is treated with an acidulant to obtain ascorbic acid. The second route is a one-step method comprising acid-catalyzed cyclization of KLG, as originally disclosed in GB Patent No. 466548 to Reichstein) and later modified by Yamazaki (Yamazaki, *J. Agri. Chem. Soc. Japan*, *28*:890-894 (1954), and *Chem. Abs.*, *50*:5992d) and again by Yodice (WO 87/00839). The Yodice method is commercially undesirable because it uses large amounts of gaseous hydrogen chloride, requires very expensive process equipment and produces an ascorbic acid product requiring extensive purification.

Lipases, a group of hydrolase enzymes, have been used with some success in the synthesis of esters of organic acids. In particular, lipases have been utilized in the transesterification of alcohols in which the esterifying agent is irreversible, such as when vinyl acetate is used as the esterifying agent (Thiel, *Catalysis Today*, 517-536 (1994)). Gutman et. al., *Tetrahedron Lett.*, *28*:3861-3864 (1987), describes a process for preparing simple 5-membered ring lactones from gamma-hydroxy methyl esters using porcine pancreatic lipase as the catalyst. However, Gutman et al., *Tetrahedron Lett.*, *28*:5367-5368 (1987), later reported that substituting delta-hydroxy methyl esters for gamma-hydroxy methyl esters and using the same catalyst produced only polymers. In EP 0 515 694 A1 to Sakashita et. al., a synthesis of esters of ascorbic acid, which are acylated on the primary hydroxyl group, comprises reacting ascorbic acid with a variety of fatty acid active esters (*i.e.*, fatty acid vinyl esters) in a polar organic solvent in the presence of a lipase.

Thus, there exists a need in the art for methods of producing (a) ascorbic acid or metal salts thereof from KLG or esters of KLG, (b) KLG from esters of KLG and (c) esters of KLG from KLG, which have high yield and high purity with little or no by-product formation and are conducted under mild conditions. Accordingly, it is to the provision of such that the present invention is primarily directed.

SUMMARY OF THE INVENTION

The present invention discloses an advancement in the chemical and biological arts in which a process for preparing ascorbic acid comprises contacting KLG or an ester of KLG with a hydrolase enzyme catalyst.

In another embodiment of the present invention, a process for producing KLG comprises contacting an ester of KLG in an aqueous solution with a hydrolase enzyme catalyst.

In still another embodiment of the present invention, a process for producing esters of KLG from KLG comprises contacting an alcoholic solution of KLG with a hydrolase enzyme catalyst. The alcoholic solution contains an alcohol corresponding to an alkyl moiety of the ester of KLG to be prepared.

In still another embodiment of the present invention, a process for producing esters of KLG from esters of KLG comprises contacting an alcoholic solution of a first ester of KLG with a hydrolase enzyme catalyst. The alcoholic solution contains an alcohol corresponding to an alkyl moiety of a second ester of KLG which is to be prepared.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the unexpected discovery that ascorbic acid can be formed from KLG or, more preferably, esters of KLG by inducing ring closure of KLG or esters of KLG using a hydrolase enzyme as a catalyst. The process for producing the ascorbic acid may be performed in the melt or in solution. The process may also be performed

in vivo or *in vitro*. For *in vivo* processes, the hydrolase enzyme catalyst may be naturally occurring within a host cell or may be introduced into a host cell or organism by recombinant DNA methods.

The present invention is also directed to the unexpected discovery that KLG can be prepared in a reversible reaction by reacting an ester of KLG in an aqueous solution using a hydrolase enzyme as a catalyst. Moreover, the present invention is directed to the unexpected discovery that an ester of KLG can be prepared by reacting KLG or another ester of KLG in an alcoholic solution using a hydrolase enzyme as a catalyst. The alcohol used to prepare the solution corresponds to the alkyl moiety of the ester of KLG being prepared.

The hydrolase enzymes for use as catalysts in the processes of the present invention may be derived from or isolated from any appropriate source organisms. Examples of which include, but are not limited to, plants, microorganisms, and animals, such as yeast, bacteria, mold, fungus, birds, reptiles, fish, and mammals. Hydrolase enzymes for the purposes of this invention are defined generally by the enzyme class E.C.3.-.-.-, as defined in *Enzyme Nomenclature* (Academic Press, 1992), and are commercially available.

Preferred hydrolase enzymes are those capable of effecting hydrolysis of molecules containing carbonyl or phosphate groups. More specifically, the preferred hydrolases are capable of effecting hydrolysis at a carbonyl

carbon bearing a heteroatom single bond. Examples of such carbonyl carbons bearing a heteroatom single bond include, but are not limited to, esters, thioesters, amides, acids, acid halides, and the like. The preferred hydrolases include the enzyme class E.C.3.1.-.-, which includes hydrolases acting on ester bonds, such as esterases and lipases; the enzyme class E.C.3.2-.-, which includes glycosidases; the enzyme class E.C.3.4-.-, which includes peptide hydrolases, such as proteases; and the enzyme class E.C.3.5.-.-, which includes amidases acting on bonds other than peptide bonds. Most preferred hydrolases include proteases, amidases, lipases, and esterases.

More preferred hydrolases contain an active site serine residue which is capable of undergoing esterification or transesterification with KLG or esters of KLG. Even more preferred are those hydrolases which contain the catalytic triad of serine, histidine and apartic acid.

Preferred proteases include those derived from bacteria of the genera *Bacillus* or *Aspergillus*. Particularly preferred proteases are those obtained from the bacteria *Bacillus licheniformis*. Preferred proteases are those containing at least 70% sequence homology with *Subtilisin*. Proteases having sequence homology with *Subtilisin* are used in the detergent industry and, therefore, are readily available. More preferred are proteases having at least 80% sequence homology with *Subtilisin*, even more preferred are proteases having at least 90% sequence homology with *Subtilisin* and, in particular, proteases having at least 95%

sequence homology to *Subtilisin*. A highly preferred protease is *Subtilisin* itself having an amino acid sequence (SEQ ID NO: 1) described by Smith et al., *J. Biol. Chem.*, *243*:2184-2191 (1968), and given below:

MMRKKSFWLG MLTAFMLVFT MAFSDSASAA QPAKNVEKDY
IVGFKSGVKT ASVKKDIIKE SGGKVDKQFR IINAAKAKLD
KEALKEVKND PDVAYVEEDH VAHALAQTVP YGIPLIKADK
VQAQGFKGAN VKVAVLDTGI QASHPDLNVV GGASFVAGEA
YNTDGNGHGT HVAGTVAALD NTTGVLGVAP SVSLYAVKVL
NSSGSGTYSG IVSGIEWATT NGMDVINMSL GGPSGSTAMK
QAVDNAYARG VVVVAAAGNS GSSGNTNTIG YPAKYDSVIA
VGAVDSNSNR ASFSSVGAEL EVMAPGAGVY STYPTSTYAT
LNGTSMASPH VAGAAALILS KHPNLSASQV RNRLSSTATY
LGSSFYYGKG LINVEAAAQ.

For the convenience of the reader, Table 1 provides a summary of amino acid shorthand used above and in the remainder of the specification.

Table 1

Amino Acid Symbol	Three-Letter Abbreviation	One-Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Also encompassed by the scope of the present invention are proteases corresponding to one to six site-specific mutants, sequence additions, and sequence deletions of the sequence given above. Even more preferred are proteases corresponding to zero to two site-specific mutants of the *Subtilisin* sequence given above.

Esterases suitable for the present invention include those obtained from pig liver extract. Preferred esterases

are those having at least 70% sequence homology with pig liver esterase having an amino acid sequence (SEQ ID NO: 2) described in Matsushima et al., *FEBS Lett.*, *293*:37 (1991), and given below:

MWLLPLVLTS LASSATWAGQ PASPPVVDTA QGRVLGKYVS
LEGLAFTQPV AVFLGVPFAK PPLGSLRFAP PQPAEPWSFV
KNTTSYPPMC CQDPVVEQMT SDLFTNFTGK ERLTLEFSED
CLYLNIYTPA DLTKRGRLPV MVWIHGGGLV LGGAPMYDGV
VLAAHENFTV VVVAIQYRLG IWGFFSTGDE HSRGNWGHLD
QVAALHWVQE NIANFGGDPG SVTIFGESFT AGGESVSVLV
LSPLAKNLFH RAISESGVAL TVALVRKDMK AAAKQIAVLA
GCKTTTSAVF TFVHCLRQKS EDELLDLTLK MKFLTLDFHG
DQRESHPFLP TVVDGVLLPK MPEEILAEKD FTFNTVPYIV
GINKQEFGWL LPTMMGFPLS EGKLDQKTAT SLLWKSYPIA
NIPEELTPVA TFTDKYLGGT DDPVKKKDLF LDLMGDVVFG
VPSVTVARQH RDAGAPTYMY EFQYRPSFSS DKFTKPKTVI
GDHGDEIFSV FGFPLLKGDA PEEEVSLSKT VMKFWANFAR
SGNPNGEGLP HWPFTMYDQE EGYLQIGVNT QAAKRLKGEE
VAFWNDLLSK EAAKKPPKIK HAEL.

Esterases more preferably have at least 80% sequence homology with the sequence of the pig liver esterase given above, even more preferably at least 90% sequence homology, especially preferred at least 95% sequence homology. Highly preferred is the pig liver esterase having the sequence given above.

Also encompassed by the scope of the present invention are esterases corresponding to one to six site-specific mutants, sequence additions, and sequence deletions of the

sequence given above. Even more preferred are esterases corresponding to zero to two site-specific mutants of the pig liver esterase sequence given above.

Preferred lipases include those isolated from pigs and other mammals, microorganisms, and plants. This includes, but is not limited to, lipases obtained from the genera *Aspergillus, Mucor, Candida, Pseudomonas, Humicola, Rhizopus, Chromobacterium, Alcaligenes, Geotricum,* and *Penicillium.* Preferred lipases also include extracellular lipases, such as cutinases. More preferred lipases have at least 70% sequence homology with *Candida Antartica* type B lipase, even more preferred have at least 80% sequence homology, still more preferred have at least 90% sequence homology, and even more preferred have at least 95% sequence homology. A highly preferred lipase is the *Candida Antartica* type B lipase itself which has an amino acid sequence (SEQ ID NO: 3) described by Uppenberg et al., *Structure, 2*:293, 453 (1994), and given below:

MKLLSLTGVA GVLATCVAAT PLVKRLPSGS DPAFSQPKSV
LDAGLTCQGA SPSSVSKPIL LVPGTGTTGP QSFDSNWIPL
STQLGYTPCW ISPPPFMLND TQVNTEYMVN AITALYAGSG
NNKLPVLTWS QGGLVAQWGL TFFPSIRSKV DRLMAFAPDY
KGTVLAGPLD ALAVSAPSVW QQTTGSALTT ALRNAGGLTQ
IVPTTNLYSA TDEIVQPQVS NSPLDSSYLF NGKNVQAQAV
CGPLFVIDHA GSLTSQFSYV VGRSALRSTT GQARSADYGI
TDCNPLPAND LTPEQKVAAA ALLAPAAAAI VAGPKQNCEP
DLMPYARPFA VGKRTCSGIV TP.

Also encompassed by the scope of the present invention are lipases corresponding to one to six site-specific mutants, sequence additions, and sequence deletions of the sequence given above. Even more preferred are lipases corresponding to zero to two site-specific mutants of the *Candida Antartica* type B sequence given above.

Preferred amidases include those isolated from bacteria of the genus *Penicillium*. A more preferred amidase has at least 80% sequence homology with *Penicillin acylase*. A particularly preferred amidase is *Penicillin acylase*, which is also referred to as *Penicillin amidohydrolase*, E.C. 3.5.1.11 (Duggleby et al., *Nature*, 373:264-268 (1995)).

For hydrolases containing serine at their active site, the first step in the reaction of either KLG or esters of KLG is believed to involve formation of a KLG-enzyme ester via acylation by KLG of the active site serine. Intra-molecular ring closure is believed to yield ascorbic acid (or its salts), whereas alcoholysis yields an ester of KLG and hydrolysis yields KLG.

The process of the present invention comprises contacting either KLG or an ester of KLG with a hydrolase enzyme to form ascorbic acid. Preferably, this reaction is performed in the presence of an organic solvent system, an aqueous solvent system or a mixture thereof. The organic solvent is preferably a C_1-C_6 alcohol. The aqueous solvent system or mixed aqueous and organic solvent systems are more preferable because ascorbic acid, KLG, and esters of KLG are generally more soluble in aqueous solvent systems. For the

in vitro production of ascorbic acid from esters of KLG, the mixed aqueous and organic solvent systems or organic solvent systems are preferable to minimize competing hydrolysis reactions which can produce KLG as a byproduct. Aqueous solvent systems are especially preferable when utilizing whole cell systems for the production of ascorbic acid *in vivo*.

In one aspect of the present invention, the ascorbic acid is produced from KLG or esters of KLG in *in vivo*, whole cell, and whole organism production systems in the presence of the hydrolase enzyme catalyst. In one embodiment, the hydrolase enzyme is naturally produced by the host organism. In another embodiment, the hydrolase enzyme is produced by the host organism through recombinant DNA technology. For example, a gene sequence encoding a hydrolase enzyme is inserted in a host organism wherein the host organism may be a microorganism, plant, or animal which is capable of expressing the hydrolase enzyme. The host organism producing the hydrolase enzyme is cultured, i.e. provided with nutrients and a suitable environment for growth, in the presence of KLG or esters of KLG to produce the ascorbic acid. Preferably, the host organism is *Pantoea citrea*, previously referred to as *Erwinia herbicola* as disclosed in U.S. Patent No. 5,008,193 to Anderson et al.

Also preferably, the host organism is one that produces KLG in addition to producing the hydrolase enzyme. Representative organisms are from the genera *Pantoea* or *Gluconobacter*, such as disclosed in Shinjoh et al., *Applied*

and Envtl. Microbiology, 61:413-420 (1995), and the genus *Corynebacterium* as disclosed in Sonoyama et al., *Applied and Envtl. Microbiology, 43*:1064-1069 (1982).

As used herein, recombinant DNA technology includes *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombinant/ genetic recombination and is well known in the art. See, for example, the techniques described in Maniatis et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1989); Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley Interscience, N.Y. (1989); Anderson et al., *Science*, 230:144-149 (1985); and U.S. Patent No. 5,441,882 to Estell et. al.

For preparations of KLG from esters of KLG, an aqueous solution of the ester of KLG is reacted with the hydrolase enzyme. A co-solvent may be used in the preparation of KLG and is preferably a C_1-C_6 alcohol.

For preparations of the esters of KLG from KLG or from other esters of KLG, the starting material is in an alcoholic solution wherein the alcohol corresponds to the alkyl moiety of the ester of KLG to be prepared. The alkyl moiety R of the alcohol ROH from which the preferred ester of KLG is derived may be chosen from branched or straight chain, saturated or unsaturated, alkyl, arylalkyls, aryls, and substituted aryls. Preferred R groups include C_1 to C_6 straight or branched chain, saturated or unsaturated alkyls. Even more preferred esters of KLG that are derived for alkyl moieties include MeKLG, ethyl-KLG, n-propyl-KLG, isopropyl-

KLG, n-butyl-KLG, isobutyl-KLG, t-butyl-KLG, and n-pentyl-KLG. The most preferred esters of KLG produced are MeKLG due to its ease of manufacture and butyl-KLG due to the advantageous use of the butanol water azeotroph in water removal. A co-solvent may be used in the preparation of the esters of KLG and is preferably water, a C_1-C_6 alcohol or a mixture thereof.

Preferred temperatures for conducting the reactions of the present invention are from 5°C to 120°C. Even more preferred temperatures are from 25°C to 100°C, and especially preferred temperatures are from 38°C to 80°C.

The preferred pH for the process of the present invention is between 1.5 and 10, and a more preferred pH is between 3 and 10. For the preparation of ascorbic acid salts from esters of KLG, a particularly preferred pH range is between 6 and 10. For the preparation of ascorbic acid as the free acid, a preferred pH is that under the pKa of ascorbic acid and, more preferred, is that under 4.2. For the preparation of KLG from esters of KLG, a particularly preferred pH range is between 5 and 10 due to the generally enhanced rates of enzyme assisted hydrolysis in this pH range. Alternatively, a pH of between 1.5 and 2.5 is particularly desirable for the generation of KLG in protonated form. Finally, for the preparation of esters of KLG from KLG, a particularly preferred pH range is between 3 and 6.

Each hydrolase has a temperature optimum, a pH optimum, and a pH and temperature range associated with activity.

Thus, the appropriate pH and temperature range for a given hydrolase is that which allows for activity of the hydrolase and avoids conditions which are denaturing or inactivating to the hydrolase. For conditions which may be denaturing, such as high temperature or the use of denaturing solvents such as methanol or the like, a minimal amount of testing may be required to define those hydrolases which remain active under a given set of conditions.

The following examples are offered by way of illustration and are not intended to limit the scope of the claimed invention.

EXAMPLES

Proton and carbon nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 300 NMR instrument operating at 300 MHZ in proton mode and 75 MHZ in carbon mode. All NMR spectra were referenced to tetramethylsilane (TMS) at 0 parts per million (ppm) and peak frequencies were recorded in ppm unless otherwise specified. HPLC (high-performance liquid chromatography) analysis was carried out using ultraviolet (UV) detection. Mass spectra (MS) were obtained using a Fisons VG Analytical Ltd. Autospec Mass Spectrometer in FD (field desorption) mode.

The KLG used in the experiments was obtained by fermentation according to the method of Lazarus et. al., Anderson et al., *Science, 230*:144-149 (1985), and was purified by concentration and crystallization. KLG may alternatively be prepared by chemical conversion from

L-sorbose according to methods well known in the art (*see e.g.*,U.S. Pat. No. 2,301,811 to Reichstein). A standard of methyl-2-keto-L-gulonate was purchased from Aldrich Chemical Company (Rare and Specialty Chemicals Catalog), in addition to being prepared by esterification of KLG by methods similar to the procedure used for the preparation of butyl-KLG, described below.

Enzyme hydrolase samples were obtained from commercial sources, including Sigma Chemical Company, Altus Biologics, Recombinant Biocatalysis, Boehringer Mannheim, Novo Nordisk, Genencor International, Thermogen, and Fluka.

Example 1

This example describes the preparation and purification of butyl 2-keto-L-gulonate.

KLG hydrate (51.62 g) was charged in a 500 ml reaction vessel under argon. The reactor was equipped with a 30.48 cm (12") vigreux column attached to a Dean Stark trap. The reactor was then charged with n-butanol (310 g) and p-toluene sulfonic acid (2.3 g). The reaction mixture was brought to reflux (81-82°C) under mild vacuum [(approximately 19.95 kPa (150 mm Hg)] with stirring. Reflux was maintained for a total of two hours and 40 minutes. Heating was discontinued. The reaction was allowed to cool and remain at room temperature for approximately 3 days. The resulting crystals were filtered through a coarse fritted glass filter and washed with two portions of n-butyl alcohol (139 g followed by 37 g). The resulting solids (24.4 g) were dissolved in

hot ethyl acetate (250 ml) and recrystallized by standing overnight at room temperature. The recrystallized butyl-KLG was isolated by filtration and dried under vacuum [0.1995 kPa (1.5 mm Hg)] until constant weight (15.97 g) was achieved.

The butyl-KLG thus prepared was found to have a solubility of at least 50 weight percent in water as it was soluble at all concentrations under 50 weight percent in water. The recrystallized butyl-KLG of this example had satisfactory proton and carbon NMR spectra and gave the predicted molecular weight by field desorption mass spectrometry.

^{1}H NMR (DMSO, digital resolution = 0.11 Hz, TMS at half height = 0.5 Hz): 6.49 (OH, d, J = 1.4 Hz), 4.96 (OH, d, J = 5.0 Hz), 4.84 (OH, d, J = 4.8 Hz), 4.78 (OH, d, J = 7.4 Hz), 4.17-4.0 (m, 2 H), 3.5-3.2 (m, approximately 5 H), 1.64-1.5 (m, 2 H), 1.4-1.35 (m, 2 H), 0.89 (CH_3, t, J = 7.3).

^{13}C NMR (DMSO, decoupled): 169.4, 96.3, 73.8, 72.8, 69.8, 64.5, 62.8, 30.0, 18.4, 13.5.

FDMS: M = 250

Example 2

The following procedure was used to demonstrate enzymes for activity under specific pH and aqueous solvent composition conditions.

Initial enzyme screens were carried out as follows. Enzyme (typically 10 mg), aqueous buffer (typically 860 microliters (ul) or 550 ul), aqueous 0.2 M $CaCl_2$ (10 ul), methanol (typically 90 ul or 400 ul), and an aqueous solution

of substrate (typically 90 ul of butyl-KLG at a typical concentration of 110,000 ppm) were added to a 2 ml polypropylene centrifuge tube. The resulting solution was vortexed briefly and placed on a shaker bath at 300 rpm at 38°C (typically for 18 hours or more). After incubation, samples were centrifuged at 14,000 G's (14,000 times gravity) for 20 minutes to remove enzyme, sampled (300 ul), and diluted to one milliliter with distilled water. If not analyzed by HPLC within the day, samples were frozen prior to analysis.

Summarized below in Table 2 is the HPLC data of the products (and remaining substrate) upon reaction of butyl-KLG (BuKLG) with a variety of enzyme hydrolases in water/methanol solution. The data were reported in terms of parts per million of KLG, MeKLG, ascorbic acid (ASA) and butyl-KLG. The reporting of a 0 (zero) indicated that the amount of material present was below the detection threshold of the instrument. Samples labeled as "no enzyme" were controls within a given run. The controls contained substrate but no enzyme and thus represented experimental and HPLC background data.

Table 2

Enzyme Screen for
Hydrolysis/Methanolysis of Butyl-KLG
(38°C for 41 Hours/38% Methanol-
Water/0.1 MES Buffer)

Enzyme	Measured, pH	KLG	MeKLG	ASA	BuKLG (ppm)
ESL-001-01	5.8	1180	2352	766	4603
ESL-001-02	5.6	704	1084	302	7736
ESL-001-03	5.7	386	527	257	8931
ESL-001-04	5.8	550	752	833	6229
ESL-001-05	5.9	456	684	469	7942
ESL-001-06	5.6	547	661	129	8896
ESL-001-07	5.7	311	755	489	6540
No Enzyme		108	325	33	10177
No Enzyme (repeat)		107	303	0	9459
No Enzyme		117	327	42	9878
No Enzyme (repeat)		103	269	2	8593
No Enzyme		116	322	0	9473

Table 2 illustrates that the hydrolases provided by Recombinant Biocatalysis (ESL-001-01 through ESL-001-07) showed appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 38% methanol-water solution buffered with morpholinoethane sulfonic acid (MES) hemisodium salt at a pH controlled between 5.5 and 6. These hydrolase enzymes are sold commercially by Recombinant Biocatalysis as recombinant esterases and lipases from thermophilic organisms under the tradename CloneZyme (trademark).

Example 3

Table 3 below illustrates that a variety of acylases, esterases, lipases, and proteases showed appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 38% methanol-water solution buffered at pH 4.8 to 5.8 with MES buffer. The enzymes labeled as ChiroClec (trademark) are crystalline crosslinked enzymes sold commercially by Altus Biologics. ChiroClec -CR is a lipase from *Candida rugosa*, ChiroClec -BL is a crystalline form of *Subtilisin* (a protease), and ChiroClec -PC is a lipase from *Pseudomonas cepacia*. *Candida Antartica* B (a lipase), pig liver esterase (a hydrolase), and *Bacillus Species* protease showed particularly high levels of activity.

Table 3

Enzyme Screen for Hydrolysis/Methanolysis of Butyl-KLG (38°C for 16 Hours/38% Methanol-Water/0.1 M MES Buffer)

Enzyme	Measured pH	KLG	MeKLG	ASA	BuKLG (ppm)
Pig Liver Esterase	5.3	446	4377	294	5711
Pseudomonas cepacia Lipase	5.3	98	295	65	11355
Porcine Pancreatic Lipase	5.4	81	316	49	10709
Candida Rugosa Lipase	5.7	122	197	180	10689
Alpha-Chymotrypsin	4.9	57	152	20	11174
Penicillin Acylase	5.6	83	1307	15	12007
Aspergillus niger Lipase	5.7	302	541	55	12290
no enzyme	5.1	88	210	5	10393
no enzyme	5.1	87	199	1	11553
Candida Antartica 'A' Lipase	5.4	88	242	37	10670
Candida lipolytica Lipase	5.3	91	92	5	11604
Candida antartica 'B' Lipase	4.8	2915	6807	0	0
Humicola lanuginosa Lipase	5	63	90	6	10191
Bacillus Species Protease	4.8	2587	5386	9	1251
no enzyme	5.2	94	194	1	11552
ChiroCLEC-CR (Dry)	5.1	113	222	2	10988
ChiroCLEC-BL (Dry)	5.4	194	642	3	5123
ChiroCLEC-PC (Pseudomonas cepacia)	5.7	147	566	1	10471
Rhizoipus Delmar Lipase	5.5	51	99	1	7392
Rhizopus Niveus Lipase	5.1	80	252	17	10453
Rhizopus Oryzae Lipase	5.5	58	172	5	10873
Chromobacterium Viscosum Lipase	5.5	433	187	1	10843
Geotricum Candidum Lipase	5	33	407	7	10000
Mucor Javanicus Lipase	5.5	33	167	97	9950
Aspergillus Oryzae Protease	5.8	289	781	96	7429
Amano-Lipase PS30(Pseudomonas)	5.3	56	300	49	9143
Amano-Lipase AK (Pseudomonas)	5.6	74	167	93	11372

Example 4

Table 4 below illustrates that a variety of acylases, esterases, lipases, and proteases showed appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 38% methanol-water solution buffered at pH 5 to 5.8 with MES buffer. Pig liver esterase, *Subtilisin Carlsberg* (a protease), *Bacillus species* protease, ChiroClec -BL, and *Candida Antartica* B lipase all show particularly high levels of activity.

Table 4

Enzyme Screen for Hydrolysis/Methanolysis of Butyl-KLG (38°C for 47.5 Hours/38% Methanol-Water/0.1 M MES Buffer)

Enzyme	Measured pH	KLG	MeKLG	ASA	BuKLG (ppm)
Pig Liver Esterase	5.3	705	2720	246	1368
Pseudomonas cepacia Lipase	5.5	77	288	46	6222
Porcine Pancreatic Lipase	5.4	229	613	222	10899
Candida rugosa Lipase	5.8	104	205	155	5417
Alpha-Chymotrypsin	5.1	82	248	54	6092
Penicillin Acylase	5.8	100	1607	30	6192
Aspergillus niger Lipase	5.3	214	391	29	6470
Mucor meihei Lipase	5.6	54	189	108	7041
ChiroCLEC-CR	5.5	115	218	99	3769
Subtilisin Carlsberg	5.1	3072	47	0	0
Candida antarctica A	5.4	166	316	35	5943
Candida lipolytica Lipase	5.7	150	166	0	6445
Candida antartica B	5.3	2210	3520	60	0
Humicola lanuginosa Lipase	5.2	129	241	42	8017
Bacillus Sp Protease	5.3	3722	1940	29	38
ChiroCLEC-BL protease	5	3744	1724	54	634
ChiroCLEC PC lipase	5.7	108	196	5	4148
Candida Rugosa esterase	5.6	70	309	61	6734
L-1 (Pseudomonas sp))	5.4	90	336	11	7066
L-2 (Candida antartica B)	5.5	2622	3764	14	913
L-3 (Candida cylindracea)	5.7	88	158	37	10343
L-5 (Candida antartica A)	5.5	153	665	42	4626
L-6 (Pseudomonas sp)	5.7	0	379	13	6183
L-7 (Porcine pancreas)	5.8	94	884	120	5488
L-8 (Humicola sp)	5.5	98	219	7	7299
no enzyme	5.6	75	234	5	5508
no enzyme	5.5	68	209	6	4968
no enzyme	5.6	65	277	16	5320

Example 5

Table 5 below illustrates that a variety of lipases and proteases showed appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 38% methanol-water solution buffered at pH 5.7 to 6.1 with MES buffer. On comparison with the other enzymes in this table, Prozyme 6 (a protease from *Aspergillus oryzae*), Protease 2A (from *Aspergillus oryzae*), and GC899 (a commercial detergent protease from Genencor International) showed higher levels of activity.

Table 5

Enzyme Screen for Hydrolysis/Methanolysis of Butyl-KLG
(38°C for 19 Hours/38% Methanol-Water/0.1 M MES Buffer)

Enzyme	Comment	Measured pH	KLG	MeKLG	ASA	BuKLG (ppm)
PS30 (Pseudomonas)	Lipase	5.9	83	213	32	10424
GC4 (Geotricum candidum)	Lipase	5.7	0	166	0	7475
AK (Pseudomonas)	Lipase	6	27	205	26	9815
G (Penicillium)	Lipase	5.8	0	0	0	9441
Newlase A (Aspergillus)	Protease	5.9	83	299	6	10368
Protease M (Aspergillus)	Protease	6	498	1054	281	6990
Prozyme 6 (Aspergillus)	Protease	6	1489	2259	0	4965
MAP10 (Mucor)	Lipase	6.1	21	148	145	8968
No enzyme		5.9	71	169	22	9463
No enzyme		5.9	75	191	6	9391
No enzyme		5.9	79	196	7	9539
D(Rhizopus)	Lipase	5.7	44	156	3	8562
Newlase II (Rhizopus)	Protease	5.9	36	164	12	9586
AY30 (Candida)	Lipase	6	0	192	33	8725
L-10 (Candida)	Lipase	5.7	0	0	0	9608
CES (Pseudomonas)	Lipase	5.8	52	296	42	9491
N (Rhizopus)	Lipase	5.8	78	404	27	9834
2A (Protease, Aspergillus)	Protease	6.1	937	1158	215	8951
Hog Pancreatic Lipase	Fluka	6	58	529	130	11114
Lipase (Sigma-1754)	Lipase	5.8	57	98	47	9845
Lipase (Sigma-1754)	Lipase	5.8	46	88	82	9428
Lipase (Sigma-8525)	Lipase	5.9	178	222	60	9041
Lipase (Sigma-1754)	Lipase	5.7	76	145	89	14257
Lipase (Sigma-3126)	Lipase	5.9	90	415	130	12756
F-15(Rhizopus)	Lipase	5.8	55	165	14	10262
Lipozyme (Novo-Liquid)	Lipase	6	82	122	160	9100
GC899 (protease)	Protease	5.8	791	2735	312	11607

Example 6

Table 6 below illustrates that a variety of lipases and proteases showed appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 8.6% methanol-water solution buffered at a pH of 5.3 to 6 with MES buffer. Protease M (*Aspergillus oryzae*), Prozyme 6 (a protease from *Aspergillus oryzae*), Protease N (*Subtilisin*), and Protease 2A (*Aspergillus oryzae*) all showed particularly high levels of activity.

Table 6

Enzyme Screen for Hydrolysis/Methanolysis of Butyl-KLG
(38° for 19 Hours/8.6% Methanol-Water/0.1 M MES)

Enzyme	Comment	Measured pH	KLG	MeKLG	ASA	BuKLG (ppm)
PS30 (Pseudomonas)	Lipase	5.9	341	163	157	8363
GC4 (Geotricum candidum)	Lipase	5.9	424	0	8	4192
AK (Pseudomonas)	Lipase	6	295	432	125	8255
G (Penicillium)	Lipase	5.8	253	323	0	7678
Newlase A (Aspergillus)	Protease	5.7	692	302	126	13408
R-10(Penicillium)	Lipase	6	527	208	583	5570
Protease M (Aspergillus)	Protease	6	3650	2262	328	1696
Prozyme 6 (Aspergillus)	Protease	5.3	7207	694	0	0
MAP10 (Mucor)	Lipase	6	369	0	231	8334
No enzyme		5.8	378	239	132	8272
No enzyme		5.8	380	205	19	8582
No enzyme		5.8	382	295	43	8785
D(Rhizopus)	Lipase	5.9	595	326	76	11656
Newlase II (Rhizopus)	Protease	5.9	323	212	28	8535
AY30 (Candida)	Lipase	5.9	330	249	254	10195
L-10 (Candida)	Lipase	5.8	302	69	55	11057
AP12 (Aspergillus)	Lipase	6	1448	738	129	7730
CES (Pseudomonas)	Lipase	5.9	197	252	0	8092
N (Rhizopus)	Lipase	6	582	348	61	9598
N (Protease, Bacillus)	Protease	5.7	1572	1289	26	1822
2A (Protease, Aspergillus)	Protease	5.7	5891	616	160	764

Hog Pancreatic Lipase	Fluka	5.8	890	791	158	5284
Lipase (Sigma-1754)	Lipase	5.9	283	116	148	6196
Lipase (Sigma-1754)	Lipase	6	348	189	415	8098
Lipase (Sigma-8525)	Lipase	6	326	93	15	4112
Lipase (Sigma-1754)	Lipase	6	300	150	154	8057
Lipase (Sigma-3126)	Lipase	5.8	787	488	99	8829
F-15(Rhizopus)	Lipase	5.9	218	124	0	8682
Lipozyme (Novo-Liquid)	Lipase	5.8	380	95	101	7251
GC899 (protease)	Protease	5.6	3354	1765	201	6991

Example 7

Table 7 below illustrates that a variety of acylases, esterases, lipases, and proteases showed appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 8.6% methanol-water solution buffered at a pH of approximately 5 to 6 with MES buffer. *Candida Antartica* B lipase, pig liver esterase, and *Bacillus species* protease showed particularly high levels of activity.

Table 7

Enzyme Screen for Hydrolysis/Methanolysis of Butyl-KLG
(38°C for 19 Hours/8.6% Methanol-Water/0.1 M MES)

Enzyme	Comment	KLG	MeKLG	ASA	BuKLG
L-1 (Pseudomonas sp))	Lipase	137	116	47	7601
L-2 (Candida antartica B)	Lipase	5249	1921	0	768
L-3 (Candida cylindracea)	Lipase	183	64	107	6920
L-4 (Pseudomonas sp)	Lipase	239	163	88	9957
L-5 (Candida antartica A)	Lipase	278	344	0	6245
L-6 (Pseudomonas sp)	Lipase	90	219	15	6613
L-7 (Porcine pancreas)	Lipase	1007	575	106	5392
L-8 (Humicola sp)	Lipase	209	70	150	7957
no enzyme		168	152	6	8753
no enzyme		152	144	3	8233
no enzyme		170	137	18	8157
ESL-001-01	Recombinant	1271	906	375	4635
ESL-001-02	Biocatalysis	883	329	332	5949
ESL-001-03	Enzymes	290	123	447	7333
ESL-001-04		511	161	306	6207
ESL-001-05		364	124	299	6402
ESL-001-06		329	117	118	6934
ESL-001-07		0	122	430	15752
Pig Liver Esterase		2726	3731	423	10
Pseudomonas cepacia Lipase		241	109	224	9135
Porcine Pancreatic Lipase		333	291	314	7888
Candida rugosa Lipase		296	86	451	8697
no enzyme		153	116	8	8234

Alpha-Chymotrypsin	protease	330	1076	65	3855
Penicillin Acylase		187	1248	157	8110
no enzyme		100	73	3	5296
no enzyme		144	113	7	8106
Aspergillus niger Lipase		479	72	84	8455
Mucor meihei Lipase		229	278	156	8620
ChiroCLEC-CR	lipase	233	155	11	7569
Subtilisin Carlsberg		4463	93	0	4428
Candida antarctica A	lipase	215	0	175	7573
Candida lipolytica Lipase		198	62	92	8445
Bacillus Sp Protease		4920	642	13	72
ChiroCLEC-BL protease		2860	1233	135	4051
ChiroCLEC PC lipase		127	62	2	5653
Candida Rugosa esterase		178	120	225	9382

Example 8

Table 8 below illustrates that a variety of acylases, esterases, lipases, and proteases showed appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 8.6% methanol-water solution buffered at a pH of approximately 5.8 to 6.2 with MES buffer. Pig liver esterase, *Candida Antartica* B lipase, *Bacillus species* protease, and lightly crosslinked crystalline *Subtilisin* (ChirClec-BL) showed particularly high levels of activity.

Table 8

Enzyme Screen for Hydrolysis/Methanolysis of Butyl-KLG
(38°C for 21 Hours/8.6% Methanol-Water/0.2M MES

Enzyme	Comment	pH	KLG	MeKLG	ASA	BuKLG (ppm)
Pig Liver Esterase		5.8	2373	4167	717	83
Pseudomonas cepacia Lipase		5.9	173	169	25	7384
Porcine Pancreatic Lipase		5.9	303	320	78	6860
Candida rugosa Lipase		5.9	260	112	271	7351
Alpha-Chymotrypsin	protease	5.9	506	1239	146	4707
Penicillin Acylase		6	176	1172	98	5392
Aspergillus niger Lipase		5.9	493	259	84	6364
Mucor meihei Lipase		5.9	243	283	54	7067
no enzyme		5.9	198	173	2	7137
no enzyme		5.9	216	153	0	7115
no enzyme		5.9	223	154	1	7319
Candida Antartica 'A' Lipase		5.9	222	142	148	6683
Candida lipolytica Lipase		6	721	123	25	6721
Candida antartica 'B' Lipase		5.9	2708	709	20	28
Humicola lanuginosa Lipase		5.9	176	129	10	7215
Bacillus Species Protease		5.8	5553	603	0	33
ChiroCLEC-CR (Dry)		6.1	229	170	2	7191
ChiroCLEC-BL (Dry)		5.9	4293	1282	6	1376
ChiroCLEC-PC (P. cepacia-Dry)		6.1	240	268	2	7539

Rhizoipus Delmar Lipase	6	178	0	0	7097
Rhizopus Niveus Lipase	6.2	178	181	61	7102
Rhizopus Oryzae Lipase	6.1	159	119	26	7611
Chromobacterium Viscosum Lipase	6	415	181	2	7275
Geotricum Candidum Lipase	6.1	146	122	6	6140
Mucor Javanicus Lipase	6.2	167	95	141	7422
Aspergillus Oryzae Protease	6.1	2193	1462	39	2904
Candida Rugosa Esterase	5.8	129	132	17	7164

Example 9

Table 9 below demonstrates the statistical reproduction of the activity detected for highly active enzymes in the preceding examples. Eight of the enzymes from the previous examples, which were identified as showing particularly high levels of activity, were compared under tight pH control. All of the previously identified enzymes with high levels of activity maintained this high level of activity on reanalysis. The enzymes exhibited appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 8.6% methanol-water solution buffered at a pH of approximately 5.6 to 6 with 0.2 M MES buffer. *Candida Antartica* B lipase, pig liver esterase, and *Bacillus species* protease showed particularly high levels of activity within this comparative example. Pig liver esterase showed a selectivity toward transesterification as well as significant conversions to ascorbic acid.

Table 9

Enzyme Screen for Hydrolysis/Methanolysis of Butyl-KLG
(38°C for 19 Hours/8.6% Metanol-Water/0.2 M MES Buffer)

Enzyme	Comment	PH	KLG	MeKLG	ASA	BuKLG (ppm)
N Protease	Protease	6	700	1166	297	5435
Candida Antartica B	Lipase	5.8	4347	2207	283	0
Pig Liver Esterase	Esterase	5.9	1947	4258	650	0
Bacillus sp Protease	Protease	5.6	5137	745	55	0
ChiroClec-BL (Dry)	Subtilisin	5.8	3485	1235	215	3045
Prozyme-6	Protease	5.8	3405	1518	73	1624
Protease M	Protease	6	554	668	271	6329
2A Protease	Protease	5.9	1585	1501	153	3954
no enzyme		6	135	149	14	8170
no enzyme		5.9	136	127	16	8418
no enzyme		6	142	133	13	8570

Example 10

Table 10 below compares the same enzymes as in Example 9 except at a higher concentration of organic solvent. *Candida Antartica* B and *Bacillus species* protease showed particularly high levels of activity in that they exhibited appreciable conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 38% methanol-water solution buffered at a pH of approximately 5.6 to 6.2 with 0.2 M MES buffer. Decreased, although still appreciable, activity is observed for pig liver esterase relative to that shown in Example 9.

Table 10

Enzyme Screen for Hydrolysis/Methanolysis of Butyl-KLG
(38°C for 19 Hours/38% Methanol-Water/0.2 M MES Buffer)

Enzyme	Comment	pH	KLG	MeKLG	ASA	BuKLG (ppm)
N Protease	Protease	5.9	176	1144	126	8153
Candida Antartica B	Lipase	5.8	1701	5710	213	199
Pig Liver Esterase	Esterase	6	203	1654	173	7030
Bacillus sp Protease	Protease	5.6	3104	4032	182	213
ChiroClec-BL (Dry)	Protease	5.8	1261	1693	102	5572
Prozyme-6	Protease	6	350	1268	47	7517
Protease M	Protease	6.2	141	408	199	9400
2A Protease	Protease	6.1	178	626	90	8666
no enzyme		6	69	221	8	9418
no enzyme		5.9	61	189	7	8790
no enzyme		6	63	203	9	9367

Example 11

Table 11 below compares the same enzymes as in Example 9 except at a pH buffered around 5.2. *Candida Antartica* B and pig liver esterase showed particularly high levels of activity in that they exhibited appreciable conversion of butyl-KLG to MeKLG and KLG in a 8.6% methanol-water solution buffered at a pH of approximately 4.9 to 5.3 with 0.2 M pyridine/pyridinium hydrochloride buffer. Decreased, although still appreciable, activity is observed for *Bacillus* species protease relative to Example 9.

Table 11

Enzyme Screen for Hydrolysis/Methanolysis of BUKLG
(38°C for ca. 19 Hours/8.6% Methanol-Water/0.2 M Pyridine/
Pyridiniuym Hydrochloride)

Enzyme	Comment	pH	KLG	MeKLG	ASA	BuKLG (ppm)
N Protease	Protease	5.2	87	237	47	8320
Candida Antartica B	Lipase	4.9	3460	3097	53	0
Pig Liver Esterase	Esterase	5.2	1613	5787	37	390
Bacillus sp Protease	Protease	5.1	1613	2473	70	3757
ChiroClec-BL (Dry)	Protease	5.1	987	1360	67	5603
Prozyme-6	Protease	5.2	700	840	7	6470
Protease M	Protease	5.3	187	357	0	8387
2A Protease	Protease	5.2	480	643	0	7523
no enzyme		5.3	97	0	153	9750
no enzyme		5.2	73	0	80	9547

Example 12

Table 12 below compares the same enzymes as in Example 11 except at a higher concentration of organic solvent. *Candida Antartica* B showed particularly high levels of activity in that it exhibited appreciable conversion of butyl-KLG to MeKLG and KLG in 38% methanol-water solution buffered at a pH of approximately 4.7 to 5.1 with 0.2 M pyridine/pyridinium hydrochloride buffer. All of the enzymes showed reduced activity relative to Examples 9 and 11.

Table 12

Enzyme Screen for Hydrolysis/Methanolysis of BuKLG
(38°C for ca. 19 Hours/H 4.9/38% Methanol-Water)

Enzyme	Comment	PH	KLG	MeKLG	ASA	BuKLG
N Protease	Protease	4.8	0	0	17	9093
Candida Antartica B	Lipase	4.7	1953	6470	0	5373
Pig Liver Esterase	Esterase	4.9	47	197	0	11750
Bacillus sp Protease	Protease	4.9	333	2113	30	10043
ChiroClec-BL (Dry)	Protease	4.9	97	447	7	10950
Prozyme-6	Protease	4.9	0	113	3	12730
Protease M	Protease	5.1	73	203	0	15887
2A Protease	Protease	5	67	150	0	13920
no enzyme		4.9	87	13	27	11753

Example 13

Table 13 below compares the same enzymes as in Examples 9 and 11 except at a pH buffered around 2.3. All enzymes tested showed reduced activity relative to Examples 9 and 11 for conversion of butyl-KLG to ascorbic acid, MeKLG, and KLG in a 8.6% methanol-water solution buffered at a pH of approximately 2.3-2.7 with 0.2 M phosphate buffer.

Table 13

Enzyme Screen for Hydrolysis/Methanolysis of BUKLG
(38°C for 20 Hours/8.6% Methanol-Water/pH 2.3 0.2 M Phosphate Buffer)

Enzyme	Comment	PH	KLG	MeKLG	ASA	BuKLG
N Protease	Protease	2.4	203	0	3	8980
Candida Antartica B	Lipase	2.4	397	323	0	8463
Pig Liver Esterase	Esterase	2.4	417	93	0	9500
Bacillus sp Protease	Protease	2.3	347	0	0	10987
ChiroClec-BL (Dry)	Protease	2.3	387	0	0	10580
Prozyme-6	Protease	2.4	440	0	0	12357
Protease M	Protease	2.6	137	333	0	12237
2A Protease	Protease	2.7	163	347	0	10600
No enzyme		2.3	487	0	0	10417
No enzyme		2.3	413	0	0	9897
No enzyme		2.3	407	0	0	9873

Example 14

Table 14 below compares the first 5 enzymes of Examples 9 and 11 at a buffered pH of about 6 in their ability to catalyze the esterification of KLG to methyl KLG (MeKLG) or their ability to catalyze ring closure of KLG to ascorbic acid. Low levels of activity are observed relative to examples 9 and 11.

Table 14

Enzyme Screen for Methanolysis of KLG
(38°C for 19 Hours/8.6% Methanol-Water/0.2 M MES Buffer)

Enzyme	Comment	pH	KLG	MeKLG	ASA	BuKLG
N Protease	Protease	6	3791	0	0	0
Candida Antartica B	Lipase	6	4258	0	0	0
Pig Liver Esterase	Esterase	6	4393	0	0	0
Bacillus sp Protease	Protease	6	4099	0	0	0
ChiroClec-BL (Dry)	Subtilisin	6.1	3270	0	0	0
no enzyme		6	4340	0	0	0
no enzyme		6	3295	0	0	0
no enzyme		6	4029	0	0	0

Example 15

Table 15 below demonstrates the production of MeKLG from KLG using *Candida Antartica* B lipase as catalyst in 8.6% aqueous methanol at a pH of 3-3.2. The buffer was chosen as a mixture of KLG and its sodium salt (approximately 1/9). The first three entries include enzyme catalyst and are the same conditions in triplicate. The second three entries also run in triplicate and are the same conditions as the first three entries except that no enzyme was present. The first three entries show significant esterification of KLG to MeKLG in the presence of *Candida Antartica* B lipase. The second three entries demonstrate that the conversion does not proceed in the absence of *Candida Antartica* B lipase.

Table 15

Enzyme Screen for Esterification of KLG

68 Hours at 38°C/8.6% Methanol in Aqueous Phase/Buffer = KLG + NaKLG

Enzyme	Comment	pH	KLG	MeKLG	ASA	BuKLG
Candida Antartica B	8.6% MeOH+KLG	3.1	9227	460	0	0
Candida Antartica B	8.6% MeOH+KLG	3.1	9303	530	0	0
Candida Antartica B	8.6% MeOH+KLG	3.2	9213	413	0	0
no enzyme	8.6% MeOH+KLG	2.9	9530	0	0	0
no enzyme	8.6% MeOH+KLG	2.9	9477	0	0	0
no enzyme	8.6% MeOH+KLG	2.9	9600	0	0	0

Example 16

This example demonstrates the slow decomposition of ascorbic acid under the conditions of HPLC analysis. HPLC sample standards were prepared by dissolving KLG, MeKLG, ascorbic acid (ASA), and butyl-KLG to the appropriate concentration in water. Samples of these standards were placed in filled and sealed vials, stored at room temperature, and analyzed periodically. The HPLC was calibrated on the area response for standards that were injected onto the HPLC as soon as possible after the preparation of the standards. Table 16 below shows the recorded responses for KLG, MeKLG, ascorbic acid, and butyl-KLG standards of 50, 100, and 500 ppm at time 0 (calibration time), at approximately 6.5 hours, and at approximately 12 hours after sample preparation.

Table 16

Time (minutes)	Amount Prepared	Amount Found KLG	MeKLG	ASA	BuKLG
0	50ppm standard	51	51.4	53.4	50.6
400		39.9	47.7	28.3	42.7
715		52	43	0	38.2
0	100 ppm standard	102	103	107	101
400		94.3	106.8	96.6	100.1
715		81.8	90.2	57.2	94.2
0	500 ppm standard	510	514	534	506
400		479	496	487	512
715		493	495	473	499

The ascorbic acid responses were non-linear over time with respect to the other standards and,

particularly, with respect to standards of 100 ppm or less. Given that the treatment for Examples 2-16 included approximately 16 hours or more at 38°C on a shaker bath prior to HPLC analysis, it follows that the actual level of ascorbic acid formed was greater than reported.

This invention has been described in detail with particular reference to preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Hubbs, John C.

(ii) TITLE OF INVENTION: Enzymatic Process for the Manufacture of Ascorbic Acid, 2-Keto-L-Gulonic Acid, and Esters of 2-Keto-L-Gulonic Acid

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Eastman Chemical Company
(B) STREET: P.O. Box 511
(C) CITY: Kingsport
(D) STATE: Tennessee
(E) COUNTRY: USA
(F) ZIP: 37662-5075

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: *
(B) COMPUTER: *
(C) OPERATING SYSTEM: *
(D) SOFTWARE: *

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: *
(B) FILING DATE: *
(C) CLASSIFICATION: *

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/017,879
(B) FILING DATE: 17-MAY-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Cheryl J. Tubach
(B) REGISTRATION NUMBER: *
(C) REFERENCE/DOCKET NUMBER: 70432

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 423-229-6189
(B) TELEFAX: 423-229-1239

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 379 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Met Arg Lys Lys Ser Phe Trp Leu Gly Met Leu Thr Ala Phe Met
1 5 10 15

Leu Val Phe Thr Met Ala Phe Ser Asp Ser Ala Ser Ala Ala Gln Pro
20 25 30

Ala Lys Asn Val Glu Lys Asp Tyr Ile Val Gly Phe Lys Ser Gly Val
35 40 45

Lys Thr Ala Ser Val Lys Lys Asp Ile Ile Lys Glu Ser Gly Gly Lys
50 55 60

Val Asp Lys Gln Phe Arg Ile Ile Asn Ala Ala Lys Ala Lys Leu Asp
65 70 75 80

Lys Glu Ala Leu Lys Glu Val Lys Asn Asp Pro Asp Val Ala Tyr Val
85 90 95

Glu Glu Asp His Val Ala His Ala Leu Ala Gln Thr Val Pro Tyr Gly
100 105 110

Ile Pro Leu Ile Lys Ala Asp Lys Val Gln Ala Gln Gly Phe Lys Gly
115 120 125

Ala Asn Val Lys Val Ala Val Leu Asp Thr Gly Ile Gln Ala Ser His
130 135 140

Pro Asp Leu Asn Val Val Gly Gly Ala Ser Phe Val Ala Gly Glu Ala
145 150 155 160

Tyr Asn Thr Asp Gly Asn Gly His Gly Thr His Val Ala Gly Thr Val
165 170 175

Ala Ala Leu Asp Asn Thr Thr Gly Val Leu Gly Val Ala Pro Ser Val
180 185 190

Ser Leu Tyr Ala Val Lys Val Leu Asn Ser Ser Gly Ser Gly Thr Tyr
195 200 205

Ser Gly Ile Val Ser Gly Ile Glu Trp Ala Thr Thr Asn Gly Met Asp
210 215 220

Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Thr Ala Met Lys
225 230 235 240

Gln Ala Val Asp Asn Ala Tyr Ala Arg Gly Val Val Val Val Ala Ala
245 250 255

Ala Gly Asn Ser Gly Ser Ser Gly Asn Thr Asn Thr Ile Gly Tyr Pro
260 265 270

Ala Lys Tyr Asp Ser Val Ile Ala Val Gly Ala Val Asp Ser Asn Ser
275 280 285

Asn Arg Ala Ser Phe Ser Ser Val Gly Ala Glu Leu Glu Val Met Ala
290 295 300

Pro Gly Ala Gly Val Tyr Ser Thr Tyr Pro Thr Ser Thr Tyr Ala Thr
305 310 315 320

Leu Asn Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Ala Ala Ala
325 330 335

Leu Ile Leu Ser Lys His Pro Asn Leu Ser Ala Ser Gln Val Arg Asn
340 345 350

Arg Leu Ser Ser Thr Ala Thr Tyr Leu Gly Ser Ser Phe Tyr Tyr Gly
355 360 365

Lys Gly Leu Ile Asn Val Glu Ala Ala Ala Gln
370 375

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 584 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Trp Leu Leu Pro Leu Val Leu Thr Ser Leu Ala Ser Ser Ala Thr
1 5 10 15

Trp Ala Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly
20 25 30

Arg Val Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Phe Thr Gln
35 40 45

Pro Val Ala Val Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly
50 55 60

Ser Leu Arg Phe Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val
65 70 75 80

Lys Asn Thr Thr Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Val
85 90 95

Glu Gln Met Thr Ser Asp Leu Phe Thr Asn Phe Thr Gly Lys Glu Arg
100 105 110

Leu Thr Leu Glu Phe Ser Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr
115 120 125

Pro Ala Asp Leu Thr Lys Arg Gly Arg Leu Pro Val Met Val Trp Ile
130 135 140

His Gly Gly Gly Leu Val Leu Gly Gly Ala Pro Met Tyr Asp Gly Val
145 150 155 160

Val Leu Ala Ala His Glu Asn Phe Thr Val Val Val Val Ala Ile Gln
165 170 175

Tyr Arg Leu Gly Ile Trp Gly Phe Phe Ser Thr Gly Asp Glu His Ser
180 185 190

Arg Gly Asn Trp Gly His Leu Asp Gln Val Ala Ala Leu His Trp Val
195 200 205

Gln Glu Asn Ile Ala Asn Phe Gly Gly Asp Pro Gly Ser Val Thr Ile
210 215 220

Phe Gly Glu Ser Phe Thr Ala Gly Gly Glu Ser Val Ser Val Leu Val
225 230 235 240

Leu Ser Pro Leu Ala Lys Asn Leu Phe His Arg Ala Ile Ser Glu Ser
245 250 255

Gly Val Ala Leu Thr Val Ala Leu Val Arg Lys Asp Met Lys Ala Ala
260 265 270

Ala Lys Gln Ile Ala Val Leu Ala Gly Cys Lys Thr Thr Thr Ser Ala
275 280 285

Val Phe Thr Phe Val His Cys Leu Arg Gln Lys Ser Glu Asp Glu Leu
290 295 300

Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu Asp Phe His Gly
305 310 315 320

Asp Gln Arg Glu Ser His Pro Phe Leu Pro Thr Val Val Asp Gly Val
325 330 335

Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu Lys Asp Phe Thr
340 345 350

Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu Phe Gly
355 360 365

Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly Lys Leu
370 375 380

Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro Ile Ala
385 390 395 400

Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Phe Thr Asp Lys Tyr
405 410 415

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
420 425 430

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
435 440 445

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
450 455 460

Arg Pro Ser Phe Ser Ser Asp Lys Phe Thr Lys Pro Lys Thr Val Ile
465 470 475 480

Gly Asp His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu
485 490 495

Lys Gly Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met
500 505 510

Lys Phe Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly
515 520 525

Leu Pro His Trp Pro Phe Thr Met Tyr Asp Gln Glu Glu Gly Tyr Leu
530 535 540

Gln Ile Gly Val Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu
545 550 555 560

Val Ala Phe Trp Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro
565 570 575

Pro Lys Ile Lys His Ala Glu Leu
580

(2) INFORMATION FOR SEQ ID NO:3

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 342 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Lys Leu Leu Ser Leu Thr Gly Val Ala Gly Val Leu Ala Thr Cys
1 5 10 15

Val Ala Ala Thr Pro Leu Val Lys Arg Leu Pro Ser Gly Ser Asp Pro
20 25 30

Ala Phe Ser Gln Pro Lys Ser Val Leu Asp Ala Gly Leu Thr Cys Gln
35 40 45

Gly Ala Ser Pro Ser Ser Val Ser Lys Pro Ile Leu Leu Val Pro Gly
50 55 60

Thr Gly Thr Thr Gly Pro Gln Ser Phe Asp Ser Asn Trp Ile Pro Leu
65 70 75 80

Ser Thr Gln Leu Gly Tyr Thr Pro Cys Trp Ile Ser Pro Pro Pro Phe
85 90 95

Met Leu Asn Asp Thr Gln Val Asn Thr Glu Tyr Met Val Asn Ala Ile
100 105 110

Thr Ala Leu Tyr Ala Gly Ser Gly Asn Asn Lys Leu Pro Val Leu Thr
115 120 125

Trp Ser Gln Gly Gly Leu Val Ala Gln Trp Gly Leu Thr Phe Phe Pro
130 135 140

Ser Ile Arg Ser Lys Val Asp Arg Leu Met Ala Phe Ala Pro Asp Tyr
145 150 155 160

Lys Gly Thr Val Leu Ala Gly Pro Leu Asp Ala Leu Ala Val Ser Ala
165 170 175

Pro Ser Val Trp Gln Gln Thr Thr Gly Ser Ala Leu Thr Thr Ala Leu
180 185 190

Arg Asn Ala Gly Gly Leu Thr Gln Ile Val Pro Thr Thr Asn Leu Tyr
195 200 205

Ser Ala Thr Asp Glu Ile Val Gln Pro Gln Val Ser Asn Ser Pro Leu
210 215 220

Asp Ser Ser Tyr Leu Phe Asn Gly Lys Asn Val Gln Ala Gln Ala Val
225 230 235 240

Cys Gly Pro Leu Phe Val Ile Asp His Ala Gly Ser Leu Thr Ser Gln
245 250 255

Phe Ser Tyr Val Val Gly Arg Ser Ala Leu Arg Ser Thr Thr Gly Gln
260 265 270

Ala Arg Ser Ala Asp Tyr Gly Ile Thr Asp Cys Asn Pro Leu Pro Ala
275 280 285

Asn Asp Leu Thr Pro Glu Gln Lys Val Ala Ala Ala Ala Leu Leu Ala
290 295 300

Pro Ala Ala Ala Ala Ile Val Ala Gly Pro Lys Gln Asn Cys Glu Pro
305 310 315 320

Asp Leu Met Pro Tyr Ala Arg Pro Phe Ala Val Gly Lys Arg Thr Cys
325 330 335

Ser Gly Ile Val Thr Pro
340

CLAIMS

What is claimed is:

1. A process for preparing ascorbic acid comprising contacting a compound selected from the group consisting of 2-keto-L-gulonic acid and an ester of 2-keto-L-gulonic acid with a hydrolase enzyme catalyst to form ascorbic acid.

2. The process of claim 1 wherein the hydrolase enzyme catalyst is selected from the group consisting of a protease, an esterase, a lipase and an amidase.

3. The process of claim 2 wherein the protease is obtained from a genera selected from the group consisting of *Bacillus* or *Aspergillus*.

4. The process of claim 3 wherein the protease is obtained from a *Bacillus licheniformis* bacteria.

5. The process of claim 4 wherein the protease has at least 70 percent sequence homology with a *Subtilisin* protease having a sequence as shown in SEQ ID NO: 1.

6. The process of claim 5 wherein the protease is the *Subtilisin* protease having the sequence as shown in SEQ ID NO: 1.

7. The process of claim 2 wherein the esterase is obtained from pig liver extract.

8. The process of claim 7 wherein the esterase has at least 70 percent sequence homology with a pig liver esterase having a sequence as shown in SEQ ID NO: 2.

9. The process of claim 8 wherein the esterase is the pig liver esterase having the sequence as shown in SEQ ID NO: 2.

10. The process of claim 2 wherein the lipase is obtained from a genera selected from the group consisting of *Aspergillus, Mucor, Candida, Pseudomonas, Humicola, Rhizopus, Chromobacterium, Alcaligenes, Geotricum* and *Penicillium*.

11. The process of claim 10 wherein the lipase obtained from the genus *Candida* is a lipase having at least 70 percent sequence homology with a *Candida Antartica* B lipase having a sequence as shown in SEQ ID NO: 3.

12. The process of claim 11 wherein the lipase is the *Candida Antartica* B lipase having the sequence as shown in SEQ ID NO: 3.

13. The process of claim 2 wherein the amidase is obtained from a genus *Penicillium*.

14. The process of claim 13 wherein the amidase has at least 80% sequence homology with a *Penicillin acylase*.

15. The process of claim 14 wherein the amidase is the *Penicillin acylase*.

16. The process of claim 1 wherein the hydrolase enzyme catalyst contains an active site serine residue.

17. The process of claim 16 wherein the hydrolase enzyme catalyst contains a catalytic triad of serine, histidine and aspartic acid.

18. The process of claim 1 wherein, prior to contacting the compound with the hydrolase enzyme catalyst, the compound is formed into a solution with a solvent.

19. The process of claim 18 wherein the solvent is selected from the group consisting of water, a C_1 to C_6 alcohol and a mixture thereof.

20. The process of claim 1 wherein contacting the compound with the hydrolase enzyme catalyst occurs at a pH between 1.5 and 10.

21. The process of claim 1 wherein contacting the compound with the hydrolase enzyme catalyst occurs at a temperature from 5°C to 120°C.

22. The process of claim 1 wherein, prior to contacting the compound with the hydrolase enzyme catalyst, the hydrolase enzyme catalyst is naturally expressed from a host organism *in vivo*.

23. The process of claim 1 wherein, prior to contacting the compound with the hydrolase enzyme catalyst, a gene sequence encoding the hydrolase enzyme catalyst is inserted into a host organism and the host organism is cultured to express the hydrolase enzyme catalyst *in vivo*.

24. The process of claim 23 wherein the host organism is *Pantoea citrea*.

25. The process of claim 22 or claim 23 wherein the host organism produces KLG.

26. A mixture containing ascorbic acid prepared according to the process of claim 1.

27. A process for preparing 2-keto-L-gulonic acid comprising the steps of:

(a) preparing an aqueous solution of an ester of 2-keto-L-gulonic acid, and

(b) then contacting the ester of 2-keto-L-gulonic acid in solution with a hydrolase enzyme catalyst to form 2-keto-L-gulonic acid.

28. The process of claim 27 wherein the hydrolase enzyme catalyst is selected from the group consisting of a protease, an esterase, a lipase and an amidase.

29. The process of claim 27 wherein a co-solvent is used in preparing the aqueous solution.

30. The process of claim 29 wherein the co-solvent is a C_1 to C_6 alcohol.

31. A process for preparing an ester of 2-keto-L-gulonic acid comprising the steps of:

(a) preparing an alcoholic solution of 2-keto-L-gulonic acid and an alcohol corresponding to an alkyl moiety of an ester of 2-keto-L-gulonic acid to be formed; and

(b) then contacting the 2-keto-L-gulonic acid in solution with a hydrolase enzyme catalyst to form the ester of 2-keto-L-gulonic acid.

32. The process of claim 31 wherein the hydrolase enzyme catalyst is selected from the group consisting of a protease, an esterase, a lipase and an amidase.

33. The process of claim 31 wherein a co-solvent is used in preparing the alcoholic solution.

34. The process of claim 33 wherein the co-solvent is selected from the group consisting of water, a C_1 to C_6 alcohol and a mixture thereof.

35. A process for preparing an ester of 2-keto-L-gulonic acid comprising the steps of:

(a) preparing an alcoholic solution of a first ester of 2-keto-L-gulonic acid and an alcohol corresponding to an alkyl moiety of a second ester of 2-keto-L-gulonic acid to be formed; and

(b) then contacting the first ester of 2-keto-L-gulonic acid in solution with a hydrolase enzyme catalyst to form the second ester of 2-keto-L-gulonic acid.

36. The process of claim 35 wherein the hydrolase enzyme catalyst is selected from the group consisting of a protease, an esterase, a lipase and an amidase.

37. The process of claim 35 wherein a co-solvent is used in preparing the alcoholic solution.

38. The process of claim 37 wherein the co-solvent is selected from the group consisting of water, a C_1 to C_6 alcohol and a mixture thereof.

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 97/08668

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12P17/04 C12P7/60

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 292 303 A (GENENTECH INC) 23 November 1988 see claims ---	1
A	EP 0 401 704 A (MITSUBISHI RAYON CO) 12 December 1990 see claims ---	1
A	EP 0 207 763 A (BIO TECH RESOURCES) 7 January 1987 see claims ---	1
A	WO 85 01745 A (KRAFT INC) 25 April 1985 see claims --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
17 September 1997	30.09.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Delanghe, L

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 97/08668

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAYES D G: "THE CATALYTIC ACTIVITY OF LIPASES TOWARD HYDROXY FATTY ACIDS - A REVIEW" JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 73, no. 5, 1 May 1996, pages 543-549, XP000588420 see the whole document	1

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/US 97/08668

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0292303 A	23-11-88	US 5008193 A	16-04-91
		AU 612672 B	18-07-91
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